DESCRIPTION

HEAT STABLE VARIANTS OF ADENOSINE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE

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Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional Application Serial No. 60/496,188, filed August 18, 2003, the disclosure of which is incorporated herein by reference in its entirety.

Background of the Invention

The sessile nature of plant life generates a constant exposure to environmental factors that exert positive and negative effects on its growth and development. One of the major impediments facing modern agriculture is adverse environmental conditions. One important factor which causes significant crop loss is heat stress. Temperature stress greatly reduces grain yield in many cereal crops such as maize, wheat, and barley. Yield decreases due to heat stress range from 7 to 35% in the cereals of world-wide importance.

A number of studies have identified likely physiological consequences of heat stress. Early work by Hunter *et al.* (1977) using growth chamber conditions showed that temperature decreased the duration of grain filling in maize. Similar results in which the duration of grain filling was adversely altered by increased temperatures were identified by Tollenaar and Bruulsema (1988). Badu-Apraku *et al.* (1983) measured a marked reduction in the yield of maize plants grown under the day/night temperature regime of 35/15 °C compared to growth in a 25/15 °C temperature regime. Reduced yields due to increased temperatures is also supported by historical as well as climatological studies (Thompson 1986; Thompson 1975; Chang 1981; Conroy *et al.*, 1994). That the physiological processes of the developing seed are adversely affected by heat stress is evident from studies using an *in vitro* kernel culture

system (Jones *et al.*, 1981; Jones *et al.*, 1984; Cheikh *et al.*, 1995). Maize kernels cultured at the above-optimum temperature of 35 °C exhibited a dramatic reduction in weight.

Work with wheat identified the loss of soluble starch synthase (SSS) activity as a hallmark of the wheat endosperm's response to heat stress (Hawker *et al.*, 1993; Denyer *et al.*, 1994; Jenner 1994). Additional studies with SSS of wheat endosperm show that it is heat labile (Rijven 1986; Keeling *et al.*, 1993; Jenner *et al.*, 1995).

ADP glucose pyrophosphorylase (AGP) is another important starch biosynthesis enzyme in plants. AGP catalyzes the conversion of ATP and α-glucose-1-phosphate to ADP-glucose and pyrophosphate. ADP-glucose is used as a glycosyl donor in starch biosynthesis by plants and in glycogen biosynthesis by bacteria. The importance of ADP-glucose pyrophosphorylase as a key enzyme in the regulation of starch biosynthesis was noted in the study of starch deficient mutants of maize (*Zea mays*) endosperm (Tsai *et al.*, 1966; Dickinson *et al.*, 1969). Biochemical and genetic evidence has identified AGP as a key enzyme in starch biosynthesis in higher plants and glycogen biosynthesis in *E. coli* (Preiss *et al.*, 1994; Preiss *et al.*, 1996). AGP catalyzes what is viewed as the initial step in the starch biosynthetic pathway with the product of the reaction being the activated glucosyl donor, ADPglucose. This is utilized by starch synthase for extension of the polysaccharide polymer (reviewed in Hannah 1996).

Initial studies with potato AGP showed that expression in *E. coli* yielded an enzyme with allosteric and kinetic properties very similar to the native tuber enzyme (Iglesias *et al.*, 1993; Ballicora *et al.*, 1995). Greene *et al.* (1996a, 1996b) showed the usefulness of the bacterial expression system in their structure-function studies with the potato AGP. Multiple mutations important in mapping allosteric and substrate binding sites have been identified (Okita *et al.*, 1996).

AGP enzymes have been isolated from both bacteria and plants. Bacterial AGP consists of a homotetramer, whereas plant AGP from photosynthetic and non-photosynthetic tissues is a heterotetramer composed of two different subunits. The plant enzyme is encoded by two different genes, with one subunit being larger than the other. This feature has been noted in a number of plants. The AGP subunits in spinach leaf have molecular weights of 54 kDa and 51 kDa, as estimated by SDS-PAGE. Both subunits are immunoreactive with antibody raised against purified AGP from spinach leaves (Copeland *et al.*, 1981; Morell *et al.*, 1988). Immunological analysis using antiserum prepared against the small and large

subunits of spinach leaf showed that potato tuber AGP is also encoded by two genes (Okita *et al.*, 1990, *supra*). The cDNA clones of the two subunits of potato tuber (50 and 51 kDa) have also been isolated and sequenced (Muller-Rober *et al.*, 1990; Nakata *et al.*, 1991). The large subunit of potato tuber AGP is heat stable (Nakata *et al.*, 1991, *supra*).

As Hannah and Nelson (1975, 1976) postulated, both *Shrunken-2* (*Sh2*) (Bhave *et al.*, 1990) and *Brittle-2* (*Bt2*) (Bae *et al.*, 1990) are structural genes of maize endosperm ADP-glucose pyrophosphorylase. *Sh2* and *Bt2* encode the large subunit and small subunit of the enzyme, respectively. Based on cDNA sequencing, *Sh2* and *Bt2* proteins have predicted molecular weight of 57,179 Da (Shaw *et al.*, 1992) and 52,224 Da, respectively. The endosperm is the site of most starch deposition during kernel development in maize. *Sh2* and *Bt2* maize endosperm mutants have greatly reduced starch levels corresponding to deficient levels of AGP activity. Mutations of either gene have been shown to reduce AGP activity by about 95% (Tsai and Nelson, 1966, *supra*; Dickinson and Preiss, 1969, *supra*). Furthermore, it has been observed that enzymatic activities increase with the dosage of functional wild type (wt) *Sh2* and *Bt2* alleles, whereas mutant enzymes have altered kinetic properties. AGP is the rate limiting step in starch biosynthesis in plants. Stark *et al.* (1992) placed a mutant form of *E. coli* AGP in potato tuber and obtained a 35% increase in starch content.

The cloning and characterization of the genes encoding the AGP enzyme subunits have been reported for various plants. These include *Sh2* cDNA (Bhave *et al.*, 1990, *supra*), *Sh2* genomic DNA (Shaw *et al.*, 1992, *supra*), and *Bt2* cDNA (Bae *et al.*, 1990, *supra*) from maize; small subunit cDNA (Anderson *et al.*, 1989) and genomic DNA (Anderson *et al.*, 1991) from rice; and small and large subunit cDNAs from spinach leaf (Morell *et al.*, 1988, *supra*) and potato tuber (Muller-Rober *et al.*, 1990, *supra*; Nakata *et al.*, 1991, *supra*). In addition, cDNA clones have been isolated from wheat endosperm and leaf tissue (Olive *et al.*, 1989) and *Arabidopsis thaliana* leaf (Lin *et al.*, 1988). AGP sequences from barley have also been described in Ainsworth *et al.* (1995).

AGP has been found to function as an allosteric enzyme in all tissues and organisms investigated to date. The allosteric properties of AGP were first shown to be important in *E. coli*. A glycogen-overproducing *E. coli* mutant was isolated and the mutation mapped to the structural gene for AGP, designated as *glyC*. The mutant *E. coli*, known as *glyC*-16, was shown to be more sensitive to the activator, fructose 1,6 bisphosphate, and less sensitive to the inhibitor, cAMP (Preiss 1984). Although plant AGP's are also allosteric, they respond to different effector molecules than bacterial AGP's. In plants, 3-phosphoglyceric acid (3-PGA)

functions as an activator while phosphate (PO_4) serves as an inhibitor (Dickinson and Preiss, 1969, *supra*).

Using an *in vivo* mutagenesis system created by the *Ac*-mediated excision of a *Ds* transposable element fortuitously located close to a known activator binding site, Giroux *et al.* (1996) were able to generate site-specific mutants in a functionally important region of maize endosperm AGP. One mutant, *Rev6,* contained a tyrosine-serine insert in the large subunit of AGP and conditioned a 11-18% increase in seed weight. Published international patent applications WO 99/58698 and WO 98/22601 and issued U.S. Patent No. 6,069,300 disclose mutations in the large subunit of maize AGP enzyme that, when expressed, confer increased heat stability to the enzyme in comparison to that observed for wild type AGP enzyme. In addition, published international application WO 01/64928 teaches that various characteristics, such as seed number, plant biomass, Harvest Index *etc.*, can be increased in plants transformed with a polynucleotide encoding a large subunit of maize AGP containing the *Rev6* mutation.

Ou-Lee and Setter (1985) examined the effects of temperature on the apical or tip regions of maize ears. With elevated temperatures, AGP activity was lower in apical kernels when compared to basal kernels during the time of intense starch deposition. In contrast, in kernels developed at normal temperatures, AGP activity was similar in apical and basal kernels during this period. However, starch synthase activity during this period was not differentially affected in apical and basal kernels. Further, heat-treated apical kernels exhibited an increase in starch synthase activity over control. This was not observed with AGP activity. Singletary *et al.* (1993, 1994) using an *in vitro* culture system quantified the effect of various temperatures during the grain fill period. Seed weight decreased steadily as temperature increased from 22-36 °C. A role for AGP in yield loss is also supported by work from Duke and Doehlert (1996). These researchers showed that transcript levels decreased to a varying degree, but only one enzyme, AGP, showed a marked decrease in activity with the lower transcript levels. They postulated that AGP may have a faster turnover rate than the other enzymes, and hence is more sensitive to changes in transcript levels. More recent work by Wilhelm *et al.* (1999) also makes a strong argument for AGP's role in yield loss during heat stress. The Wilhelm *et al.* authors studied seven inbreds over three replications, and through Q_{10} analysis, showed that AGP was the only enzyme that exhibited lower activity than the control.

Work by Keeling *et al.* (1993, *supra*) quantified SSS activity in maize and wheat using Q_{10} analysis, and showed that SSS is an important control point in the flux of carbon into starch. *In vitro* biochemical studies with AGP and SSS clearly show that both enzymes of maize are heat labile. Maize endosperm AGP loses 96% of its activity when heated at 57 °C for five minutes (Hannah *et al.*, 1980). This is in contrast to potato AGP which is fully stable at 70 °C (Sowokinos *et al.*, 1982; Okita *et al.*, 1990). Although the small subunits of AGP are highly conserved among a variety of plant species (Hannah *et al.*, 2001), the N-termini of potato tuber and maize endosperm small subunits exhibit sequence differences. Heat inactivation studies with SSS showed that it is also labile at higher temperatures, and kinetic studies determined that the *Km* value for amylopectin rose exponentially when temperature increased from 25-45 °C (Jenner *et al.*, 1995, *supra*).

Brief Summary of the Invention

The subject invention concerns polynucleotides encoding a small subunit of a plant AGP enzyme that has one or more mutations in the amino acid sequence of the subunit protein, wherein the mutation confers increased heat stability to an AGP enzyme when the mutant small subunit forms part of the enzyme. As provided herein, amino acid changes in the N-terminus of the small subunit of heat labile plant AGP results in AGP enzymes that are significantly more heat stable in that the mutant AGP retains significant levels of enzymatic activity following exposure to heat treatment compared to wild type AGP. In one embodiment, the polynucleotide encodes a mutant small subunit of maize AGP.

The subject invention also concerns mutant AGP small subunit polypeptides encoded by polynucleotides of the present invention. AGP enzymes that comprise a mutant small subunit are also contemplated by the invention.

The subject invention also concerns methods for providing a plant with increased resistance to heat conditions. Plants with heat labile AGP can be transformed with or bred to contain a polynucleotide of the present invention. The subject invention also concerns transformed plant cells, plant tissue, and plants and transgenic progeny thereof.

Brief Description of the Figures

Figure 1 shows the alignment of the N-termini of the potato tuber (Pss) and maize endosperm (Mss) small subunits of AGP. The amino acid number is given in parentheses to

the right of the subunit name. The amino acids in bold are those under evaluation in this study.

Figure 2 shows the percent heat stability with various mutations in the maize endosperm AGP small subunit. The results are the average of at least two independent experiments. Each individual experiment contained triplicates of each sample. The percent heat stability is measured by comparing the amount of activity of each sample remaining after heat treatment with the amount of activity obtained before heating. See Table 2 and Materials and Methods for assay conditions.

Figures 3A-3C show reactions carried out at pH 7.0. Reactions were performed in duplicate and were started with 0.15 μg of purified enzyme. Reactions proceeded for 15 minutes and were denatured in a boiling water bath for 2 minutes. **Figure 3A** shows results as the log of the specific activity verses time. **Figures 3B-3C** show blue native gel of time points from part A. T is the ~220 kD tetramer, D is the ~100kD dimer and M represents the ~50kD monomer.

Brief Description of the Sequences

SEQ ID NO:1 is a polynucleotide sequence encoding a wild type maize endosperm AGP small subunit polypeptide.

SEQ ID NO:2 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:1.

SEQ ID NO:3 is a polynucleotide sequence encoding a mutant maize endosperm AGP small subunit polypeptide of the present invention.

SEQ ID NO:4 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:3 of the present invention.

SEQ ID NO:5 is a polynucleotide sequence encoding a mutant maize endosperm AGP small subunit polypeptide of the present invention.

SEQ ID NO:6 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:5 of the present invention.

SEQ ID NO:7 is a polynucleotide sequence encoding a mutant maize endosperm AGP small subunit polypeptide of the present invention.

SEQ ID NO:8 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:7 of the present invention.

SEQ ID NO:9 is a polynucleotide sequence encoding a mutant maize endosperm AGP small subunit polypeptide of the present invention.

SEQ ID NO:10 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:9 of the present invention.

SEQ ID NO:11 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide comprising a *Rev6* mutation.

SEQ ID NO:12 is a polypeptide having an amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:11.

SEQ ID NO:13 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide comprising a *Rev6* mutation and an *HS33* mutation.

SEQ ID NO:14 is a polypeptide encoded by the polynucleotide sequence of SEQ ID NO:13.

SEQ ID NO:15 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS33*.

SEQ ID NO:16 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:15.

SEQ ID NO:17 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS13*.

SEQ ID NO:18 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:17.

SEQ ID NO:19 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS14*.

SEQ ID NO:20 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:19.

SEQ ID NO:21 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS16*.

SEQ ID NO:22 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:21.

SEQ ID NO:23 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS40*.

SEQ ID NO:24 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:23.

SEQ ID NO:25 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS47*.

SEQ ID NO:26 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:25.

SEQ ID NO:27 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS RTS 48-2*.

SEQ ID NO:28 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:27.

SEQ ID NO:29 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS RTS 60-1*.

SEQ ID NO:30 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:29.

SEQ ID NO:31 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS 33F*.

SEQ ID NO:32 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:31.

SEQ ID NO:33 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS33M*.

SEQ ID NO:34 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:33.

SEQ ID NO:35 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS7+3*.

SEQ ID NO:36 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:35.

SEQ ID NO:37 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS6+3*.

SEQ ID NO:38 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:37.

SEQ ID NO:39 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS7+6*.

SEQ ID NO:40 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:39.

SEQ ID NO:41 is a polynucleotide sequence encoding a mutant maize endosperm AGP large subunit polypeptide of the present invention designated herein as *HS7+6+3*.

SEQ ID NO:42 is a polypeptide having the amino acid sequence encoded by the polynucleotide sequence of SEQ ID NO:41.

Detailed Disclosure of the Invention

The subject invention concerns polynucleotides encoding a small subunit of a plant AGP enzyme having one or more mutations in the amino acid sequence wherein the mutation confers increased heat stability to the expressed AGP enzyme. Mutations in the N-terminus of the small subunit of heat labile plant AGP provide for AGP enzymes that are significantly more heat stable compared to wild type AGP in that the mutant AGP retains significant levels of enzymatic activity following exposure to heat treatment.

In one embodiment, a polynucleotide of the invention encodes a mutant small subunit of maize AGP. In a further embodiment, a polynucleotide of the invention encodes a maize endosperm AGP small subunit comprising an amino acid mutation wherein the tyrosine at position 36 of the wild type sequence is changed to an amino acid that when expressed as an AGP enzyme confers increased heat stability on the enzyme. In an exemplified embodiment, a polynucleotide of the present invention encodes a maize endosperm AGP small subunit polypeptide comprising an amino acid mutation wherein the tyrosine at position 36 of the wild type sequence is changed to a cysteine. In one embodiment, the polynucleotide encodes a maize endosperm AGP small subunit polypeptide having an amino acid sequence shown in SEQ ID NO:4, or a functional fragment or variant thereof. In a specific embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NO:4 comprises the nucleotide sequence shown in SEQ ID NO:3, or a functional fragment or variant thereof.

In a further exemplified embodiment, a polynucleotide of the invention encodes a maize endosperm AGP small subunit polypeptide comprising an amino acid mutation wherein the tyrosine at amino acid position 36 is changed to a cysteine and, in addition, a glutamine residue is inserted between the serine at amino acid position 34 and the threonine at amino acid position 35 of the wild type AGP small subunit sequence. In one embodiment, the polynucleotide encodes a maize endosperm AGP small subunit polypeptide having an amino acid sequence shown in SEQ ID NO:8, or a functional fragment or variant thereof. In a specific embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ

ID NO:8 comprises the nucleotide sequence shown in SEQ ID NO:7, or a functional fragment or variant thereof.

In a still further exemplified embodiment, a polynucleotide of the invention encodes a maize endosperm AGP small subunit polypeptide comprising an amino acid mutation wherein the tyrosine at position 36 of the wild type sequence is changed to a cysteine and, in addition, a glutamic acid residue is inserted between the serine at amino acid position 34 and the threonine at amino acid position 35 of the wild type sequence. In one embodiment, the polynucleotide encodes a maize endosperm AGP small subunit polypeptide having an amino acid sequence shown in SEQ ID NO:10, or a functional fragment or variant thereof. In a specific embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NO:10 comprises a nucleotide sequence shown in SEQ ID NO:9, or a functional fragment or variant thereof.

Because of the homology of AGP polypeptides between various species of plants (Smith-White *et al.*, 1992), the ordinarily skilled artisan can readily determine the position for mutations in an AGP small subunit from plants other than maize that correspond to the position of mutations in maize AGP as disclosed herein, and can prepare polynucleotides encoding mutations in the small subunits of AGP of other plants that correspond to the mutations of the present invention exemplified in maize endosperm AGP small subunit sequences. Thus, the present invention encompasses polynucleotides that encode a mutant small subunit of AGP of plants other than maize, including, but not limited to, wheat, barley, oats, and rice, that confers increased heat stability when expressed in the plant.

The subject invention also concerns polynucleotides encoding a mutant small subunit of the invention and also encoding a large subunit of a plant AGP enzyme. The subject invention also concerns nucleic acid compositions comprising i) a polynucleotide encoding a mutant small subunit of the invention, and ii) a polynucleotide encoding a large subunit of a plant AGP enzyme. The large subunit in any of the embodiments of the present invention can have a wild type sequence or the large subunit can comprise one or more mutations that confer increased heat stability to an AGP enzyme containing the mutant large subunit. Polynucleotide sequences encoding mutant large subunits of maize AGP having increased heat stability include SEQ ID NOs:15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, and 41. Polynucleotides can comprise coding and non-coding regions or polynucleotides can comprise the coding only sequences, for example, nucleotides 10 through 1563 of SEQ ID NO:15. Mutant large subunits of maize AGP having increased heat stability include SEQ ID

NOs:16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, and 42. The large subunit can also comprise mutations that confer increased individual seed weight on a plant expressing an AGP enzyme containing the mutant large subunit. Mutations in the large subunit of a plant AGP that increase heat stability or that increase individual seed weight of a plant have been described in U.S. Patent Nos. 6,069,300; 5,589,618; 5,650,557; 6,403,863; and 5,872,216 and in published international applications WO 99/58698; WO 98/22601; WO 03/0070901; WO 98/10082; and WO 02/072784.

The subject invention also concerns polynucleotide expression constructs comprising a polynucleotide sequence of the present invention encoding a mutant small subunit of AGP that when present in a functional AGP enzyme confers increased heat stability to the enzyme. In one embodiment, an expression construct of the invention comprises a polynucleotide sequence encoding a maize endosperm AGP small subunit polypeptide comprising an amino acid sequence selected from SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:10, or a functional fragment or variant thereof. In a specific embodiment, the polynucleotide sequence comprises a polynucleotide sequence selected from SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, or a functional fragment or variant thereof. Expression constructs comprising a polynucleotide sequence encoding a mutant small subunit of AGP can also optionally comprise a polynucleotide sequence encoding a wild type or mutant large subunit of AGP. Expression constructs of the invention generally include regulatory elements that are functional in the intended host cell in which the expression construct is to be expressed in. Thus, a person of ordinary skill in the art can select regulatory elements for use in bacterial host cells, yeast host cells, plant host cells, insect host cells, mammalian host cells, and human host cells. Regulatory elements include promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements. As used herein, the term "expression construct" refers to a combination of nucleic acid sequences that provides for transcription of an operably linked nucleic acid sequence. As used herein, the term "operably linked" refers to a juxtaposition of the components described wherein the components are in a relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation.

An expression construct of the invention can comprise a promoter sequence operably linked to a polynucleotide sequence encoding a mutant AGP small subunit of the invention. Promoters can be incorporated into a polynucleotide using standard techniques known in the art. Multiple copies of promoters or multiple promoters can be used in an expression

construct of the invention. In a preferred embodiment, a promoter can be positioned about the same distance from the transcription start site as it is from the transcription start site in its natural genetic environment. Some variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the expression construct.

If the expression construct is to be provided in or introduced into a plant cell, then plant viral promoters, such as, for example, a cauliflower mosaic virus (CaMV) 35S (including the enhanced CaMV 35S promoter (see, for example U.S. Patent No. 5,106,739)) or a CaMV 19S promoter can be used. Other promoters that can be used for expression constructs in plants include, for example, prolifera promoter, Ap3 promoter, heat shock promoters, T-DNA 1'- or 2'-promoter of *A. tumafaciens*, polygalacturonase promoter, chalcone synthase A (CHS-A) promoter from petunia, tobacco PR-1a promoter, ubiquitin promoter, actin promoter, alcA gene promoter, pin2 promoter (Xu *et al.*, 1993), maize WipI promoter, maize trpA gene promoter (U.S. Patent No. 5,625,136), maize CDPK gene promoter, and RUBISCO SSU promoter (U.S. Patent No. 5,034,322) can also be used. Seed-specific promoters such as the promoter from a β-phaseolin gene (of kidney bean) or a glycinin gene (of soybean), and others, can also be used. Constitutive promoters (such as the CaMV, ubiquitin, actin, or NOS promoter), tissue-specific promoters (such as the E8 promoter from tomato), developmentally-regulated promoters, and inducible promoters (such as those promoters than can be induced by heat, light, hormones, or chemicals) are also contemplated for use with polynucleotide expression constructs of the invention.

For expression in animal cells, an expression construct of the invention can comprise suitable promoters that can drive transcription of the polynucleotide sequence. If the cells are mammalian cells, then promoters such as, for example, actin promoter, metallothionein promoter, NF-kappaB promoter, EGR promoter, SRE promoter, IL-2 promoter, NFAT promoter, osteocalcin promoter, SV40 early promoter and SV40 late promoter, Lck promoter, BMP5 promoter, TRP-1 promoter, murine mammary tumor virus long terminal repeat promoter, STAT promoter, or an immunoglobulin promoter can be used in the expression construct. The baculovirus polyhedrin promoter can be used with an expression construct of the invention for expression in insect cells.

For expression in prokaryotic systems, an expression construct of the invention can comprise promoters such as, for example, alkaline phosphatase promoter, tryptophan (trp)

promoter, lambda P_L promoter, β-lactamase promoter, lactose promoter, phoA promoter, T3 promoter, T7 promoter, or tac promoter (de Boer *et al.*, 1983).

Promoters suitable for use with an expression construct of the invention in yeast cells include, but are not limited to, 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase promoter, metallothionein promoter, alcohol dehydrogenase-2 promoter, and hexokinase promoter.

Expression constructs of the invention may optionally contain a transcription termination sequence, a translation termination sequence, a sequence encoding a signal peptide, and/or enhancer elements. Transcription termination regions can typically be obtained from the 3' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. A signal peptide sequence is a short amino acid sequence typically present at the amino terminus of a protein that is responsible for the relocation of an operably linked mature polypeptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Targeting gene products to an intended cellular and/or extracellular destination through the use of an operably linked signal peptide sequence is contemplated for use with the polypeptides of the invention. Classical enhancers are cis-acting elements that increase gene transcription and can also be included in the expression construct. Classical enhancer elements are known in the art, and include, but are not limited to, the CaMV 35S enhancer element, cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element. Intron-mediated enhancer elements that enhance gene expression are also known in the art. These elements must be present within the transcribed region and are orientation dependent. Examples include the maize shrunken-1 enhancer element (Clancy and Hannah, 2002).

DNA sequences which direct polyadenylation of mRNA transcribed from the expression construct can also be included in the expression construct, and include, but are not limited to, an octopine synthase or nopaline synthase signal. The expression constructs of the invention can also include a polynucleotide sequence that directs transposition of other genes, *i.e.*, a transposon.

Expression constructs can also include one or more dominant selectable marker genes, including, for example, genes encoding antibiotic resistance and/or herbicide-resistance for selecting transformed cells. Antibiotic-resistance genes can provide for resistance to one or

more of the following antibiotics: hygromycin, kanamycin, bleomycin, G418, streptomycin, paromomycin, neomycin, and spectinomycin. Kanamycin resistance can be provided by neomycin phosphotransferase (NPT II). Herbicide-resistance genes can provide for resistance to phosphinothricin acetyltransferase or glyphosate. Other markers used for cell transformation screening include genes encoding β-glucuronidase (GUS), β-galactosidase, luciferase, nopaline synthase, chloramphenicol acetyltransferase (CAT), green fluorescence protein (GFP), or enhanced GFP (Yang *et al.*, 1996).

The subject invention also concerns polynucleotide vectors comprising a polynucleotide sequence of the invention that encodes a mutant plant AGP small subunit. Unique restriction enzyme sites can be included at the 5' and 3' ends of an expression construct or polynucleotide of the invention to allow for insertion into a polynucleotide vector. As used herein, the term "vector" refers to any genetic element, including for example, plasmids, cosmids, chromosomes, phage, virus, and the like, which is capable of replication when associated with proper control elements and which can transfer polynucleotide sequences between cells. Vectors contain a nucleotide sequence that permits the vector to replicate in a selected host cell. A number of vectors are available for expression and/or cloning, and include, but are not limited to, pBR322, pUC series, M13 series, and pBLUESCRIPT vectors (Stratagene, La Jolla, CA).

Polynucleotides of the present invention can be composed of either RNA or DNA. Preferably, the polynucleotides are composed of DNA. The subject invention also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode mutant AGP small subunit polypeptides of the present invention. Therefore, all sequences that encode a mutant AGP small subunit of the invention are contemplated within the scope of the invention. In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, small subunit AGP proteins of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional activity of the polypeptide encoded by the polynucleotides of the present

invention. Allelic variants of the nucleotide sequences encoding a small subunit of AGP of the invention are also encompassed within the scope of the invention.

The subject invention also concerns mutant AGP small subunit polypeptides wherein the mutation(s) confers increased heat stability to an AGP enzyme that comprises the mutant small subunit relative to wild type AGP enzyme. The mutant polypeptides can be encoded by polynucleotides of the invention. In an exemplified embodiment, a maize endosperm AGP small subunit polypeptide of the present invention comprises an amino acid mutation wherein the tyrosine at position 36 of the wild type sequence is changed to a cysteine. In a specific embodiment, the maize endosperm AGP small subunit polypeptide comprises an amino acid sequence shown in SEQ ID NO:4, or a functional fragment or variant thereof. In another exemplified embodiment, a maize endosperm AGP small subunit polypeptide of the present invention comprises an amino acid mutation wherein a tyrosine at amino acid position 36 is changed to a cysteine and, in addition, a glutamine residue is inserted between the serine at amino acid position 34 and the threonine at amino acid position 35 of the wild type sequence. In a specific embodiment, the maize endosperm AGP small subunit polypeptide comprises an amino acid sequence shown in SEQ ID NO:8, or a functional fragment or variant thereof. In a further exemplified embodiment, a maize endosperm AGP small subunit polypeptide of the present invention comprises an amino acid mutation wherein the tyrosine at position 36 of the wild type sequence is changed to a cysteine and, in addition, a glutamic acid residue is inserted between the serine at amino acid position 34 and the threonine at amino acid position 35 of the wild type sequence. In a specific embodiment, the maize endosperm AGP small subunit polypeptide comprises an amino acid sequence shown in SEQ ID NO:10, or a functional fragment or variant thereof. The polypeptides of the invention can be in isolated or purified form.

Polypeptide fragments according to the subject invention typically comprise a contiguous span of about or at least 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182,

183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, or 475 amino acids of SEQ ID NO:4 (474 amino acids would be the largest fragment size for SEQ ID NO:4 since the full-length sequence is 475 amino acids), SEQ ID NO:8, or SEQ ID NO:10.

Polypeptide fragments of the subject invention can be any integer in length from at least about 25 consecutive amino acids to 1 amino acid less than the sequence shown in SEQ ID NO:4, SEQ ID NO:8, or SEQ ID NO:10. Thus, for SEQ ID NO:4, a polypeptide fragment can be any integer of consecutive amino acids from about 25 to 474 amino acids. The term "integer" is used herein in its mathematical sense and thus representative integers include: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207,

208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, and/or 475.

Each polypeptide fragment of the subject invention can also be described in terms of its N-terminal and C-terminal positions. For example, combinations of N-terminal to C-terminal fragments of about 25 contiguous amino acids in length to fragments that are one (1) amino acid less than the full length polypeptide of SEQ ID NO:4, SEQ ID NO:8, or SEQ ID NO:10 are included in the present invention. Thus, using SEQ ID NO:4 as an example, a 25 consecutive amino acid fragment could correspond to amino acids of SEQ ID NO:4 selected from the group consisting of 1-25, 2-26, 3-27, 4-28, 5-29, 6-30, 7-31, 8-32, 9-33, 10-34, 11-35, 12-36, 13-37, 14-38, 15-39, 16-40, 17-41, 18-42, 19-43, 20-44, 21-45, 22-46, 23-47, 24-48, 25-49, 26-50, 27-51, 28-52, 29-53, 30-54, 31-55, 32-56, 33-57, 34-58, 35-59, 36-60, 37-61, 38-62, 39-63, 40-64, 41-65, 42-66, 43-67, 44-68, 45-69, 46-70, 47-71, 48-72, 49-73, 50-74, 51-75, 52-76, 53-77, 54-78, 55-79, 56-80, 57-81, 58-82, 59-83, 60-84, 61-85, 62-86, 63-87, 64-88, 65-89, 66-90, 67-91, 68-92, 69-93, 70-94, 71-95, 72-96, 73-97, 74-98, 75-99, 76-100, 77-101, 78-102, 79-103, 80-104, 81-105, 82-106, 83-107, 84-108, 85-109, 86-110, 87-111, 88,-112, 89-113, 90-114, 91-115, 92-116, 93-117, 94-118, 95-119, 96-120, 97-121, 98-122, 99-123, 100-124, 101-125, 102-126, 103-127, 104-128, 105-129, 106-130, 107-131, 108-132, 109-133, 110-134, 111-135, 112-136, 113-137, 114-138, 115-139, 116-140, 117-141, 118-142, 119-143, 120-144, 121-145, 122-146, 123-147, 124-148, 125-149, 126-150, 127-151, 128-152 and so on. Similarly, the amino acids corresponding to all other

fragments of sizes between 26 consecutive amino acids and 474 consecutive amino acids of SEQ ID NO:4 (or 475 for SEQ ID NO:8 and SEQ ID NO:10) are included in the present invention and can also be immediately envisaged based on these examples. Therefore, additional examples, illustrating various fragments of the polypeptides of SEQ ID NO:4, SEQ ID NO:8, or SEQ ID NO:10 are not individually listed herein in order to avoid unnecessarily lengthening the specification.

Polypeptide fragments comprising:

a) 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, or 475 consecutive amino acids of SEQ ID NO:4, SEQ ID NO:8, or SEQ ID NO:10 may alternatively be described by the formula "n to c" (inclusive), where "n" equals the N-

terminal amino acid position and "c" equals the C-terminal amino acid position of the polypeptide. In this embodiment of the invention, "n" is an integer having a lower limit of 1 and an upper limit of the total number of amino acids of the full length polypeptide minus 24 (*e.g.*, 475-24=451 for SEQ ID NO:4). "c" is an integer between 25 and the number of amino acids of the full length polypeptide sequence (475 for SEQ ID NO:4) and "n" is an integer smaller than "c" by at least 24. Therefore, for SEQ ID NO:4, "n" is any integer selected from the list consisting of: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451; and "c" is any integer selected from the group consisting of: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92,

93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, and 475, provided that "n" is a value less than "c" by at least 24. Every combination of "n" and "c" positions are included as specific embodiments of polypeptide fragments of the invention. Examples illustrating the various fragments of a polypeptide contemplated under this formula are not individually listed in order to avoid unnecessarily lengthening the specification. However, all embodiments of a particular polypeptide can be immediately envisaged from the description provided herein. All ranges used to describe any polypeptide fragment embodiment of the present invention are inclusive unless specifically set forth otherwise.

Substitution of amino acids other than those specifically exemplified or naturally present in a plant AGP small subunit of the invention are also contemplated within the scope of the present invention. For example, non-natural amino acids can be substituted for the amino acids of an AGP small subunit, so long as the AGP small subunit protein having the

substituted amino acids retains substantially the same biological activity as the AGP small subunit protein in which amino acids have not been substituted. Examples of non-natural amino acids include, but are not limited to, ornithine, citrulline, hydroxyproline, homoserine, homocysteine, phenylglycine, taurine, iodotyrosine, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ-amino butyric acid, ε-amino hexanoic acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, norleucine, norvaline, sarcosine, homocitrulline, cysteic acid, τ-butylglycine, τ-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, C-methyl amino acids, N-methyl amino acids, and amino acid analogues in general. Non-natural amino acids also include amino acids having derivatized side groups. Furthermore, any of the amino acids in the protein can be of the D (dextrorotary) form or L (levorotary) form. Allelic variants of a protein sequence of an AGP small subunit used in the present invention are also encompassed within the scope of the invention.

Amino acids can be generally categorized in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an AGP small subunit protein of the present invention having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the AGP small subunit protein having the substitution still retains substantially the same biological activity as the AGP small subunit protein that does not have the substitution. Polynucleotides encoding an AGP small subunit protein having one or more amino acid substitutions in the sequence are contemplated within the scope of the present invention. Table 1 below provides a listing of examples of amino acids belonging to each class.

Table 1.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

The subject invention also concerns variants of the polynucleotides of the present invention that encode biologically-active mutant AGP small subunit proteins of the invention. Variant sequences include those sequences wherein one or more nucleotides of the sequence have been substituted, deleted, and/or inserted. The nucleotides that can be substituted for natural nucleotides of DNA have a base moiety that can include, but is not limited to, inosine, 5-fluorouracil, 5-bromouracil, hypoxanthine, 1-methylguanine, 5-methylcytosine, and tritylated bases. The sugar moiety of the nucleotide in a sequence can also be modified and includes, but is not limited to, arabinose, xylulose, and hexose. In addition, the adenine, cytosine, guanine, thymine, and uracil bases of the nucleotides can be modified with acetyl, methyl, and/or thio groups. Sequences containing nucleotide substitutions, deletions, and/or insertions can be prepared and tested using standard techniques known in the art.

Fragments and variants of an AGP small subunit of the present invention can be generated as described herein and tested for the presence of enzymatic function using standard techniques known in the art. For example, for testing fragments and/or variants of an AGP small subunit, the small subunit can be expressed in conjunction with an AGP large subunit to form an AGP heterotetramer enzyme and the enzyme assayed by way of a "forward" assay, *i.e.*, assaying for the conversion of ATP and α-glucose-1-phosphate to ADP-glucose and pyrophosphate, or by way of a "reverse" assay according to the present invention. AGP-enzymes comprising a fragment and/or variant of an AGP small subunit of the invention can also be subjected to heat treatment, *e.g.*, 55-60 °C for several minutes, prior to enzymatic assay in order to test for increased heat stability of the enzyme. Thus, an ordinarily skilled artisan can readily prepare and test fragments and variants of an AGP small subunit of the invention and determine whether the fragment or variant retains functional enzymatic activity and/or confers heat stability relative to full-length or a non-variant AGP small subunit.

Polynucleotides and polypeptides contemplated within the scope of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those sequences of the invention specifically exemplified herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87,

88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. *See* NCBI/NIH website.

The subject invention also contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the polynucleotide sequences exemplified herein so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis *et al.*, 1982). As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25 C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature, Tm, is described by the following formula (Beltz *et al.*, 1983):

Tm=81.5 C+16.6 Log[Na+]+0.41(%G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20 C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

As used herein, the terms "nucleic acid" and "polynucleotide" refer to a deoxyribonucleotide, ribonucleotide, or a mixed deoxyribonucleotide and ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides. The polynucleotide sequences include the DNA strand sequence that is transcribed into RNA and the strand sequence that is complementary to the DNA strand that is transcribed. The polynucleotide sequences also include both full-length sequences as well as shorter sequences derived from the full-length sequences. Allelic

variations of the exemplified sequences also fall within the scope of the subject invention. The polynucleotide sequence includes both the sense and antisense strands either as individual strands or in the duplex.

The subject invention also concerns cells transformed with a polynucleotide of the present invention encoding a mutant AGP small subunit of the invention. The subject invention also concerns cells transformed with a nucleic acid composition of the present invention. In one embodiment, the cell is transformed with a polynucleotide sequence comprising a sequence encoding the amino acid sequence shown in any of SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:10, or a functional fragment or variant thereof. In a specific embodiment, the cell is transformed with a polynucleotide sequence shown in any of SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, or a functional fragment or variant thereof. Preferably, the polynucleotide sequence is provided in an expression construct of the invention. The transformed cell can be a prokaryotic cell, for example, a bacterial cell such as *E. coli* or *B. subtilis*, or the transformed cell can be a eukaryotic cell, for example, a plant cell, including protoplasts, or an animal cell. Plant cells include, but are not limited to, dicotyledonous, monocotyledonous, and conifer cells. Animal cells include human cells, mammalian cells, avian cells, and insect cells. Mammalian cells include, but are not limited to, COS, 3T3, and CHO cells.

Plants, plant tissues, and plant cells transformed with or bred to contain a polynucleotide of the invention or a nucleic acid composition of the invention are also contemplated by the present invention. Plants, plant tissues, and plant cells that contain an AGP enzyme comprising a mutant small subunit of the invention and, optionally, a mutant AGP large subunit that confers increased heat stability and/or increased seed weight for a plant is also contemplated within the scope of the invention. Plants and plant tissue expressing the mutant polynucleotides of the invention exhibit increased heat stability when subjected to heat stress during development. Increased heat stability of plants can provide for increased yields from those plants, particularly under conditions of heat stress. Plants within the scope of the present invention include monocotyledonous plants, such as, for example, rice, wheat, barley, oats, sorghum, maize, sugarcane, pineapple, onion, bananas, coconut, lilies, turfgrasses, and millet. In a particularly preferred embodiment, the plant is a cereal. Cereals to which this invention applies include, for example, maize, wheat, rice, barley, oats, rye, and millet. Preferably, the plant, plant tissue, or plant cell is *Zea mays*. Plants within the scope of the present invention also include dicotyledonous plants, such as, for example, peas,

alfalfa, tomato, melon, chickpea, chicory, clover, kale, lentil, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, grape, cotton, sunflower, and lettuce; and conifers. Techniques for transforming plant cells with a gene are known in the art and include, for example, *Agrobacterium* infection, biolistic methods, electroporation, calcium chloride treatment, *etc.* Transformed cells can be selected, redifferentiated, and grown into plants using standard methods known in the art. The seeds and progeny of any transformed or transgenic plant cells or plants of the invention are also included within the scope of the present invention.

Plants can also be bred to contain a polynucleotide to express a mutant small subunit of the invention. In addition, a plant having a polynucleotide of the invention in its genome can be bred with a plant that expresses a mutant heat stable and/or phosphate insensitive large subunit of AGP and progeny selected that express an AGP enzyme comprising a mutant small subunit of the invention and the mutant large subunit from the parent plants. Methods for breeding and selecting for plants having the desired characteristics are known in the art.

The subject invention also concerns methods for providing a plant with increased resistance to heat stress or elevated temperatures by incorporating a polynucleotide of the present invention in the genome of the plant cells and expressing the polypeptide encoded by the polynucleotide. In one embodiment, a plant is grown from the plant cells. Preferably, the polynucleotide encodes a mutant AGP small subunit derived from the same plant species as the plant. In one embodiment, the plant is maize. In a specific embodiment, a polynucleotide encoding an amino acid sequence shown in SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:10, or a functional fragment or variant thereof, is incorporated into a maize plant genome. In a specific embodiment, the polynucleotide comprises a nucleotide sequence shown in SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, or a functional fragment or variant thereof. Methods of the invention also contemplate incorporating a polynucleotide that encodes a mutant large subunit of AGP that comprises a mutation conferring heat stability and/or phosphate insensitivity into the genome of a plant cell that comprises a polynucleotide of the invention and expressing the mutant large subunit encoded by the polynucleotide to provide a mutant AGP enzyme of the invention.

The subject invention also concerns AGP enzymes that comprise heat stable mutants of the small subunit of AGP of the present invention combined with large subunits of AGP, including wild type and heat stable mutants of the large subunit of AGP. The mutant subunits can be provided as fragments or variants as described herein. The AGP enzymes of

the invention can be in isolated or purified form. Mutants of the large subunit of AGP that confer heat stability to an AGP enzyme can also be readily prepared and are described in U.S. Patent No. 6,069,300 and published international applications WO 99/58698 and WO 98/22601. Polynucleotide sequences encoding mutant large subunits of maize AGP having increased heat stability include SEQ ID NOs:15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, and 41. Mutant large subunits of maize AGP having increased heat stability include SEQ ID NOs:16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, and 42. Heat stable mutants of the large subunit can be co-expressed with the mutant small subunits of the present invention to further enhance the stability of an AGP enzyme. The subject invention also contemplates AGP enzymes that comprise mutant small subunits of the present invention combined with a mutant large subunit that confers increased individual seed weight when expressed in an AGP enzyme in a plant, such as the *Rev6* mutation. Mutant large subunits of maize AGP that confer increased individual seed weight include polypeptides comprising the amino acid sequence shown in SEQ ID NO:12. Polynucleotides encoding mutant large subunits that confer increased individual seed weight include SEQ ID NO:11. The combination of a mutant small subunit of the invention having a heat stabilizing mutation, and a mutant large subunit having a heat stabilizing mutation such as, for example, *HS 33* or *HS 40*, and a mutation conferring increased seed weight, *e.g.*, *Rev 6*, in a large subunit of maize AGP is specifically contemplated in the present invention. Mutant large subunits of maize AGP having heat stability and conferring increased individual seed weight include polypeptides comprising the amino acid sequence shown in SEQ ID NO:14. Polynucleotides encoding mutant large subunits that have heat stability and confer increased individual seed weight include SEQ ID NO:13. U.S. Patent Nos. 5,589,618 and 5,650,557 disclose polynucleotides (*e.g.*, *Rev6*) that encode mutations in the large subunit of AGP that confer increased seed weight in plants that express the mutant polypeptide. The subject invention also concerns AGP enzymes that comprise heat stable small subunit mutants of the present invention and mutant large subunits as described in International patent Application No. PCT/US01/06622, which was published on September 7, 2001 as WO 01/64928.

Materials and Methods

Site-directed Mutagenesis. Mutations in the maize endosperm small subunit were created essentially as described by Horton *et al.* (1993). The maize endosperm AGP small subunit is encoded by the gene *brittle-2 (Bt2)*. Construct STCL (SEQ ID NO:3) encodes a

maize AGP small subunit protein (SEQ ID NO:4) having a change from a tyrosine at amino acid position 36 of the wild type sequence to a cysteine. Construct QTYL (SEQ ID NO:5) encodes a maize AGP small subunit protein (SEQ ID NO:6) having an insertion of a glutamine between the serine at amino acid position 34 and the threonine at amino acid position 35 of the wild type sequence. The QTCL construct (SEQ ID NO:7) encodes a maize AGP small subunit protein (SEQ ID NO:8) having a change from a tyrosine at amino acid position 36 to a cysteine and an insertion of a glutamine between the serine at amino acid position 34 and the threonine at amino acid position 35. Another construct, ETCL (SEQ ID NO:9), encodes a maize AGP small subunit protein (SEQ ID NO:10) having a glutamic acid insertion between the serine at amino acid position 34 and the threonine at amino acid position 35, and the change from a tyrosine to a cysteine at position 36. The mutations were verified by sequence analysis.

Plasmids and Bacterial Strains. DNA fragments created from mutagenic PCR of the maize endosperm small subunit were digested with Nco I and Kpn I. These digested fragments were used to replace the equivalent wild type region of *Bt2* in an expression vector. The vector was transformed into the *Escherichia coli* strain AC70R1-504 which also contained the wild type *shrunken-2 (Sh2)* coding region on a compatible expression vector (Giroux *et al.*, 1996). *Sh2* encodes the large subunit of AGP. The SH2 and BT2 proteins can polymerize to form active heterotetrameric AGP. The AC70RI-504 cell line contains a mutation which renders the strain incapable of producing bacterial AGP (Iglesias *et al.*, 1993).

Growth and Purification of Maize AGP from *E.coli*. Protein inductions were as described by Greene and Hannah (1998) with a few modifications. *E. coli* strain AC70R1-504, which lacks the functional AGP gene and cannot synthesize glycogen, was transformed with both pMoncSh2 and pMoncBt2 (plasmids containing the large and small subunit of wt-AGP respectively). An overnight culture was grown with constant shaking at 225 rpm at 37 °C in LB media containing 75 ug/ml spectinomycin and 50 μg/ml kanamycin. An aliquot of the overnight culture (12.5 ml) was used to inoculate a 1L flask containing the same media. The 1L flask was grown until the OD_{600} reached 0.5-0.6. The cultures were cooled to room temperature and protein expression was induced by the addition of 0.2mM isopropyl-beta-D-thiogalactoside (IPTG) and 0.02mg/ml nalidixic acid. Expression continued for 3 hours at room temperature with constant shaking. Cells were centrifuged at 8000 x g supernatant removed and stored as pellets at –80 °C.

<u>Preparation of Extracts</u>. For crude extracts, the bacterial pellets were resuspended in 1.0 ml of extraction buffer (50 mM HEPES, pH 7.5, 200 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA and 5% Sucrose) with 20% ammonium sulfate, 50 μg/ml lysozyme, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml chymostatin, and 1 mM benzamidine added. The lysate was maintained on ice and sonicated three times for ten seconds each. The sample was centrifuged for five minutes at 12,500 rpm at 4 °C and the supernatant was transferred to a new tube on ice. Solid ammonium sulfate was added to 45% saturation and the sample was centrifuged for five minutes at 12,500 rpm at 4 °C. The pellet was resuspended in extraction buffer containing protease inhibitors and stored on ice. The concentration of the crude protein extract was determined using the Bio-Rad Protein Assay Using BSA as a standard.

<u>Assay A (forward direction, radioactive)</u>. AGP activity of the crude extracts were determined in the direction of ADP-glucose synthesis as described in Burger *et al.* (2003) with the only modifications being a decrease in the reaction time to five minutes. AGP activity in the direction of Glucose-1-Phosphate (G-1-P) synthesis is essentially as described in Kleczkowski *et al.* (1993) with a reduction in scale. Nanomoles of product are calculated by generating a standard curve with G-1-P. Both the forward and reverse reactions were started by the addition of the enzyme. For heat treatments of the crude extract, the enzymes were diluted to 1.0 μg/μl and divided into two tubes. A single tube remained on ice while the second tube was placed at 58 °C for 6 minutes with occasional gentle agitation. The value reported within an experiment is the average from triplicate samples.

<u>Assay B (reverse direction, radioactive)</u>. A non-radioactive endpoint assay was used to determine the amount of glucose-1-phosphate produced by coupling it to NADH production using phosphoglucomutase and glucose–6-phosphate dehydrogenase. The temperature of all the assays was 37 °C unless otherwise specified. Standard reaction mixtures contained 100m M MOPS HCl pH 7.4, 0.4 mg/ml BSA, 5mM $MgCl_2$, 1mM ADP-Glucose, 20mM 3-P Phosphoglyceric Acid, 1mM Sodium Pyrophosphate and enzyme in 100 μl reaction volume. Reactions were incubated at 37 °C for 5 minutes and terminated by boiling in a water bath for 1 minute. After reaction termination, 330 μl of water was added to the reaction mixture followed by 70 μl of a development mix containing a final concentration of 100m M MOPS HCl pH 7.4, 0.1 mg/ml BSA, 7mM $MgCl_2$, 0.6mM NAD, 1U Glucose-6-Phosphate dehydrogenase, and 1U Phosphoglucomutase. Reactions were centrifuged for 5 minutes and then the absorbance read at 340 nm. The amount of G-1-P produced in each

assay was calculated based on a standard curve using freshly prepared G-1-P instead of enzyme. All assay tubes were pre-warmed to 37 °C prior to assaying. All assays were initiated by the addition of enzyme. Specific activity is defined as a unit/mg protein. Purification was always monitored using the reverse assay.

Assay C (forward reaction, nonradioactive). A non-radioactive endpoint assay was used to determine the amount of PPi produced by coupling it to a decrease in NADH using pyrophosphate reagent (Sigma P-7275). Standard reaction mixtures contained 50 mM HEPES pH 7.0, 15mM MgCl2, 4.0 mM ATP, and 4.0 mM Glucose-1-Phosphate in a total volume of 200 µl. The 3-Phosphoglyceric acid (3-PGA) was added at varying amounts, as specified. When varied, the substrates ranged from 0-5mM. Reactions were terminated after 5 minutes by boiling in a water bath for 1 minute. The reactions were developed by adding 300 µl of Pyrophosphate reagent (1 bottle diluted to 22.5 ml with water) to each assay and then the absorbance read at 340 nm. The change in absorbance between the blank and the reaction was used to calculate the amount to PPi produced for each sample. All reactions were linear with time and enzyme concentration. All assay tubes were pre-warmed to 37 °C prior to assaying and were initiated by the addition of enzyme.

Enzyme kinetics. To determine the extent of activation with or with out 3-PGA, 0.1 or 2.0 µg respectively, of purified maize wt-AGP was incubated for 12.5 minutes in the forward assay. To determine the activation constant for the maize wt AGP, 0.2 µg of purified maize wt-AGP was incubated for 12.5 minutes in the forward assay. 3-PGA concentrations ranged from 0-5.0 mM. Curves were fit using Graph Pad Prism using non-linear regression. At this enzyme concentration no activity can be detected in the absence of 3-PGA.

Native protein gels. The 1/2 life of wt and QTCL at 42 °C was determined by desalting enzyme in 50 mM HEPES, pH 6.5, 5.0 mM $MgCl_2$, 0.5 mM EDTA. Heat was applied to desalted enzyme (0.15mg/ml) and at the appropriate time, enzyme was withdrawn from the tube and placed on ice. This enzyme was then divided for use in activity assays and blue native gels. All reactions were carried out using Assay B with 10mM 3-PGA. The blue native gels were prepared as outlined on World Wide Website: amershambiosciences.com under the heading of gradient gels. The gradient used was 5-18%. Two types of cathode buffer were prepared, one contained 0.002% coomassie and the other without coomassie. Aminocaproic acid was not used in the gel buffer. The gels were run at 4 °C for 20 minutes at 100V in cathode buffer containing coomassie then the voltage was increased to 200V for

an additional 20 minutes. Finally, the gel was transferred to cathode buffer without coomassie and run at 200V until the dye front was off the gel. The gel was equilibrated in cold 1X Transfer Buffer (25mM Tris Base, 192mM Glycine, and 20% Methanol) + 1% SDS.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1—Enzymatic Activity and Heat Stability of Mutant AGP

Point mutations were created in the N-terminus of the maize endosperm small subunit, *brittle 2* (Bt2) and expressed with wild type large subunit, Shrunken 2, to form a mutant maize AGP enzyme. The mutant enzyme was assayed for increased heat stability relative to wild type maize endosperm AGP. All the modified BT2 proteins tested exhibit activity levels comparable to the wild type maize endosperm before heat treatment; however, the QTCL mutant has a slight increase. Since the specific activity is consistent among the preparations (Table 2), there is no change in enzyme turnover or expression levels caused by the mutations. The most dramatic increases in activity over wild type maize endosperm are observed after heat treatments of 58 °C for six minutes. The change of tyrosine to a cysteine results in a dramatic increase in heat stability (see Figure 2, Table 3). In the case of QTCL, greater than 50% of the enzyme remains active after heat treatment while only 2% of the wild type is active. The addition of glutamine by itself does not confer heat stability to the protein; however, it contributes to an increase in overall activity of the protein.

Table 2. Percentage of specific activity of small subunit mutations compared to the wild type maize endosperm AGP

Sample	Forward Assay	Reverse Assay
WT-STYL (SEQ ID NO:2)	100	100
ETCL (SEQ ID NO:10)	123 ± 25	130 ± 0
QTCL (SEQ ID NO:8)	170 ± 18	165 ± 7
STCL (SEQ ID NO:4)	135 ± 5	150 ± 5
QTYL (SEQ ID NO:6)	120 ± 5	135 ± 21

Table 2: The results are the averages of at least two experiments. All experiments contained triplicates of each sample. The forward activity was measured in the direction of ADP-glucose synthesis in the presence of 10mM 3-PGA. The reverse assay measures the amount of glucose-1-phosphate produced by conversion to NADH through a series of secondary reactions. The amount of NADH present was quantified using a spectrophotometer. All assays were done from crude extracts of *E.coli* expressed proteins.

Table 3. Percent Heat Stability of small subunit mutations

Sample	Forward Assay	Reverse Assay
WT Maize (SEQ ID NO:2)	2.4% ± 0.8	0.9% ± 1.2
ETCL (SEQ ID NO:10)	31.7% ± 2.5	44.5% ± 17.7
QTCL (SEQ ID NO:8)	50.0% ± 7.2	68.5% ± 0.7
STCL (SEQ ID NO:4)	44.3% ± 1.5	55.0% ± 5.7
QTYL (SEQ ID NO:6)	1.7% ± 1.1	1.4% ± 2.0

Table 3: The results are the average of at least two independent experiments. Each individual experiment contained triplicates of each sample. The percent heat stability is measured by comparing the amount of activity of each sample remaining after heat treatment with the amount of activity obtained before heating. See Table 2 and Materials and Methods for assay conditions.

Example 2—Kinetic Analysis

Due to the increased activity levels and greater heat stability, the QTCL mutant was chosen for further kinetic analysis. The QTCL mutant and wild type enzymes were purified from *E.coli* as described in the materials and methods. The kinetic constants, Km and Vmax for ATP and Glucose-1-Phosphate (G-1-P) were determined for both the wild type and the QTCL enzyme (Table 4). The kinetic constants for wild type, 0.066mM for ATP and 0.036mM for G-1-P, are similar to those reported in the literature. The Km values for the QTCL mutant are also similar to those obtained for the wild type enzyme. The kinetic constants determined for the forward reaction show that the mutation is not interfering with the binding of substrates. Only slight changes are seen in the Km for both ATP and G-1-P. The catalytic efficiency (Kcat/ Km) is also similar for both enzymes.

Maize endosperm AGP can be activated by the presence of 3-PGA and inactivated in the presence of Pi. The activation rate has been measured from *E.coli* expressed AGP and several genotypes of corn. The 3-PGA activation rate tends to vary between 3- to 20-fold,

which may be a reflection of different genotypes, pH of the assay or purity levels of the extracted enzyme. Since 3-PGA and Pi have an antagonistic relationship, the ratio of the two determines the rate of activation or inhibition. The rate of activation and de-activation of the QTCL mutant enzyme was compared to wild type enzyme grown in *E.coli*. The results are presented in Table 5.

The Ka for the QTCL enzyme is approximately 2-fold higher than that of wild type AGP. This data shows that the QTCL mutant enzyme is slightly less sensitive to 3-PGA levels. The extent of phosphate de-activation was determined by varying the phosphate concentration while the 3-PGA concentration remained fixed at 2.5 mM. A comparison of the Ki's reveals that the QTCL enzyme is more susceptible to phosphate inhibition than wild type. This data is consistent with PO_4- being a deactivator of the enzyme. It has been recognized by many groups that there is a direct correlation between 3-PGA activation and PO_4- de-activation. It is easier for the PO_4- to inhibit the QTCL enzyme since the experiment has only approximately 2.5X the saturating amount of 3-PGA. However, the wild type AGP is approximately 5X saturated with 3-PGA so the PO_4- is less likely to de-activate the enzyme at this 3-PGA concentration.

Table 4. Kinetic Values for purified QTCL and WT

	ATP			Glucose-1-Phosphate		
	K_m (mM)	V_{max} (µmol/min/mg)	K_{cat}/K_m	K_m (mM)	V_{max} (µmol/min/mg)	K_{cat}/K_m
WT (SEQ ID NO:2)	0.066 ± 0.01	9.25 ± 0.32	1.2 x 10^6	0.036 ± 0.008	5.5 ± 0.29	1.3 x 10^6
QTCL (SEQ ID NO:8)	0.14 ± 0.01	23.4 ± 0.53	1.4 x 10^6	0.041 ± 0.008	16.45 ± 0.82	3.4 x 10^6

Table 4: The enzymes were purified as described in materials and methods. All reactions were performed in the presence of 10mM 3-PGA. The reactions were performed in triplicate and started by the addition of 0.15 µg of purified enzyme. The reactions were incubated for 10 minutes at 37 °C then terminated by boiling for two minutes.

Table 5. Activation and Inhibition of QTCL and WT		
	3-PGA	Pi
	K_a	K_i
WT (SEQ ID NO:2)	0.57 ± 0.055	1.67
QTCL (SEQ ID NO:8)	1.08 ± 0.13	0.4

Table 5: All assays were performed in the forward direction (Assay C) using standard reaction conditions. The K_a for 3-PGA was determined by adding varying amounts of the effector from 0-3mM. The value of the Ki for Pi was calculated in the presence of 2.5 mM 3-PGA. The reactions were incubated for 10 minutes at 37 °C then terminated by boiling for two minutes. The reactions were performed in triplicate and started by the addition of 0.15 µg of purified enzyme. The curves were created using Graph Pad Prism with non-linear regression.

Example 3—Analysis of Purified AGP Enzyme

The data from the crude extracts shows that the QTCL mutant conferred more heat stability than the wild type enzyme. However, to obtain a more detailed view of the inactivation kinetics, the $t_{1/2}$ was calculated from a graph of log % activity versus time for both the wild type AGP and the QTCL mutant (Figures 3A-3C). Purified wild type and mutant AGP enzymes were incubated at 42°C for varying amounts of time then evaluated for AGP activity and protein structure. Although the purified QTCL enzyme conferred some degree of heat stability at much higher temperatures (data not shown), 42 °C was chosen because the wild type enzyme dies rapidly at elevated temperatures. At various time points, a fraction of the enzyme was withdrawn and placed on ice. The sample was divided and a portion was used for both enzymatic and structural analysis. The enzymatic data show a linear response with time (Figure 3A). The half-life of the QTCL variant and wt AGP were shown to be 12.0 min and 1.25 min respectively. The half-life of the enzyme is increased approximately 10-fold over wt AGP at this temperature. Blue native gels were then used to indicate the multi-meric state of the enzyme at the given time points (Figures 3B-3C). Initially, both wild type and QTCL have a high percentage of heterotetramers. The QTCL protein is almost exclusively in the heterotetrameric state. However, following a five-minute heat treatment the wild type heterotetramer has a higher percentage of dimers and monomers. By 20 minutes, activity cannot be detected for the wild type enzyme and the protein has completely formed an un-resolved aggregate. In contrast, the enzyme containing the QTCL

mutant subunit remains predominantly as a heterotetramer, even after a 30-minute heat treatment.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

References

U.S. Patent No. 5,625,136

U.S. Patent No. 6,069,300

U.S. Patent No. 5,034,322

U.S. Patent No. 5,106,739

U.S. Patent No. 5,589,618

U.S. Patent No. 5,650,557

U.S. Patent No. 6,403,863

U.S. Patent No. 5,872,216

WO 98/10082

WO 98/22601

WO 99/58698

WO 01/64928

WO 02/072784

WO 03/0070901

Ainsworth, C., Hosein, F., Tarvis, M., Weir, F., Burrell, M., Devos, K.M., Gale, M.D. (1995) "Adenosine Diphosphate Glucose Pyrophosphorylase Genes in Wheat: Differential Expression and Gene Mapping" *Planta* 197:1-10.

Altschul, S. F. *et al.* (1990) "Basic Local Alignment Search Tool" *J. Mol. Biol.* 215:402-410.

Altschul, S. F. *et al.* (1997) "Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs" *Nucl. Acids Res.* 25:3389-3402.

Anderson, J.M., J. Hnilo, R. Larson, T.W. Okita, M. Morell, J. Preiss (1989) "The Encoded Primary Sequence of a Rice Seed ADP-glucose Pyrophosphorylase Subunit and its Homology to the Bacterial Enzyme" *J. Biol. Chem.* 264:12238-12242.

Anderson, J.M., R. Larson, D. Landencia, W.T. Kim, D. Morrow, T.W. Okita, J. Preiss (1991) "Molecular Characterization of the Gene Encoding a Rice Endosperm-Specific ADPglucose Pyrophosphorylase Subunit and its Developmental Pattern of Transcription" *Gene* 97:199-205

Badu-Apraku, B., Hunter, R. B., Tollenaar, M. (1983) "Effect Of Temperature During Grain Filling On Whole Plant And Grain Yield In Maize (*Zea mays* L.)" *Can. J. Plant. Sci.* 63:357-363.

Bae, J.M., M. Giroux, L.C. Hannah (1990) "Cloning And Characterization Of The *Brittle-2* Gene Of Maize" *Maydica* 35:317-322.

Ballicora, M.A., Laughlin, M.J., Fu, Y., Okita, T.W., Barry, G.F., Preiss, J. (1995) "Adenosine 5'-Diphosphate-Glucose Pyrophosphorylase from Potato Tuber" *Plant Physiol.* 109:245-251.

Ballicora, M.A., Fu, Y., Frueauf, J. B., Preiss, J. (1999) "Heat Stability of the Potato Tuber ADP-Glucose Pyrophosphorylase: Role of Cys Residue 12 in the Small Subunit" *Biochem. Biophys. Res. Comm.* 257:782-786.

Beltz, G. A., Jacobs, K. A., Eickbush, T. H., Cherbas, P. T., Kafatos, F. C. (1983) "Isolation of multigene families and determination of homologies by filter hybridization methods" *Methods of Enzymology*, R. Wu, L. Grossman and K. Moldave [eds.] Academic Press, New York 100:266-285.

Bhave, M.R., S. Lawrence, C. Barton, L.C. Hannah (1990) "Identification and Molecular Characterization of *Shrunken-2* cDNA Clones of Maize" *Plant Cell* 2:581-588.

Burger, B.T., Cross J.M., Shaw, J.R., Caren,J.R., Greene,T.W., Okita, T.W., Hannah, L.C. (2003) "Relative turnover numbers of maize endosperm and potato tuber ADP-glucose pyrophosphorylases in the absence and presence of 3-PGA" *Planta* 217:449-456.

Chang, J. (1981) "Corn Yield In Relation To Photoperiod, Night Temperature, And Solar Radiation" *Agricul. Metero.* 24:253-262.

Cheikh, N., Jones, R.J. (1995) "Heat Stress Effects on Sink Activity of Developing Maize Kernels Grown In Vitro" *Physiol. Plant.* 95:59-66.

Clancy, M. and Hannah, L.C. (2002) "Splicing of the maize Sh1 first intron is essential for enhancement of gene expression, and a T-rich motif increases expression without affecting splicing," *Plant Physiol.* 130(2):918-29.

Conroy, J. P., Seneweera, S., Basra, A. S., Rogers, G., and Nissen-Wooller, B. (1994) "Influence of Rising Atmospheric CO_2 Concentrations and Temperature on Growth, Yield and Grain Quality of Cereal Crops" *Aust. J. Plant Physiol.* 21:741-758.

Copeland, L., J. Preiss (1981) "Purification of Spinach Leaf ADPglucose Pyrophosphorylase" *Plant Physiol.* 68:996-1001.

de Boer, H. A., Comstock, L. J., Vasser, M. (1983) "The tac promoter: a functional hybrid derived from the trp and lac promoters" *Proc. Natl. Acad. Sci. USA* 80(1):21-25.

Denyer, K., Hylton, C. M., Smith, A. M. (1994) "The Effect of High Temperature on Starch Synthesis and the Activity of Starch Synthase" *Aust. J. Plant Physiol.* 21:783-789.

Dickinson, D.B., J. Preiss (1969) "Presence of ADP-Glucose Pyrophosphorylase in Shrunken-2 and Brittle-2 Mutants of Maize Endosperm" *Plant Physiol.* 44:1058-1062.

Duke, E.R. Doehlert, D.C. (1996) "Effects of heat stress on enzyme activities and transcript levels in developing maize kernels grown in culture" *Environ. Exp. Botany.* 36:199-208.

Giroux, M.J., Shaw, J., Barry, G., Cobb, G.B., Greene, T., Okita, T.W., Hannah, L. C. (1996) "A Single Gene Mutation That Increases Maize Seed Weight" *Proc. Natl. Acad. Sci.* 93:5824-5829.

Greene, T.W., Chantler, S.E., Kahn, M.L., Barry, G.F., Preiss, J., Okita, T.W. (1996a) "Mutagenesis of the Potato ADPglucose Pyrophosphorylase and Characterization of an Allosteric Mutant Defective in 3-phosphoglycerate Activation" *Proc. Natl. Acad. Sci.* 93:1509-1513.

Greene, T.W., Woodbury, R.L., Okita, T. W. (1996b) "Aspartic Acid 413 Is Important for the Normal Allosteric Functioning of ADP-Glucose Pyrophosphorylase" *Plant Physiol.* 112:1315-1320.

Greene, T.W., Hannah, L.C. (1998) "Enhanced stability of maize endosperm ADP-glucose pyrophosphorylase is gained through mutants that alter subunit interactions" *Proc. Natl. Acad. Sci. USA* 95: 13342-13347.

Hannah, L.C., Tuschall, D.M., Mans, R.J. (1980) "Multiple forms of maize endosperm ADP-glucose pyrophosphorylase and their control of shrunken-2 and brittle-2" *Genetics* 95:961-970.

Hannah, L. Curtis (1996) "Starch synthesis in the maize endosperm," In: *Advances in Cellular and Molecular Biology of Plants*, Vol. 4. B. A. Larkins and I. K. Vasil (eds.). Cellular and Molecular Biology of Plant Seed Development. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Hannah, L.C., O.E. Nelson (1975) "Characterization of Adenosine Diphosphate Glucose Pyrophosphorylases from Developing Maize Seeds" *Plant Physiol.* 55:297-302.

Hannah, L.C., Nelson, Jr., O.E. (1976) "Characterization of ADP-Glucose Pyrophosphorylase from *Shrunken-2* and *Brittle-2* Mutants of Maize" *Biochem. Genet.* 14:547-560.

Hannah, L. C., Shaw, J. R., Giroux, M., Reyss, A., Prioul, J.-L. Bae, J.-M., Lee, J.-Y. (2001) "Maize genes encoding the small subunit of ADP-glucose pyrophosphorylase" *Plant Physiol.* 127:173-183.

Hawker, J. S. and Jenner, C. F. (1993) "High Temperature Affects the Activity of Enzymes in the Committed Pathway of Starch Synthesis in Developing Wheat Endosperm" *Aust. J. Plant Physiol.* 20:197-209.

Horton, Robert M., Ho, Steffan H., Pullen, Jeffery K., Hunt, Henry D., Cai, Zeling, Pease, Larry R. (1993) "Gene Splicing by Overlap Extension" In Wu, Ray ed, Methods of Enzymology: Recombinant DNA, part H, volume 217. Academic Press, New York, pp 270-279.

Hunter, R. B., Tollenaar, M., Breuer, C. M. (1977) *Can. J. Plant Sci.* 57:1127-1133.

Iglesias, A., Barry, G.F., Meyer, C., Bloksberg, L., Nakata, P., Greene, T., Laughlin, M.J., Okita, T.W., Kishore, G.M., Preiss, J. (1993) "Expression of the Potato Tuber ADP-glucose Pyrophosphorylase in *Escherichia coli*" *J. Biol Chem.* 268:1081-86

Jenner, C. F. (1994) "Starch Synthesis in the Kernel of Wheat Under High Temperature Conditions" *Aust. J. Plant Physiol.* 21:791-806.

Jenner, C. F., Denyer, K., Guerin, J. (1995) "Thermal Characteristics of Soluble Starch Synthase from Wheat Endosperm" *Aust. J. Plant Physiol.* 22:703-709.

Jones, R.J., Gengenbach, B.G., and Cardwell, V.B. (1981) "Temperature Effects On In Vitro Kernel Development of Maize" *Crop Science* 21:761-766.

Jones, R.J., Ouattar, S., and Crookston, R.K. (1984) "Thermal Environment During Endosperm Cell Division And Grain Filling In Maize: Effects On Kernel Growth And Development In Vitro" *Crop Science* 24:133-137.

Karlin S. and Altschul, S. F. (1990) "Methods for Assessing the Statistical Significance of Molecular Sequence Features by Using General Scoring Schemes" *Proc. Natl. Acad. Sci. USA* 87:2264-2268.

Karlin S. and Altschul, S. F. (1993) "Applications and Statistics for Multiple High-Scoring Segments in Molecular Sequences" *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Keeling, P.L., Bacon, P.J., Holt, D.C. (1993) "Elevated Temperature Reduces Starch Deposition in Wheat Endosperm by Reducing the Activity of Soluble Starch Synthase" *Planta.* 191:342-348.

Kleczhowski, L.A., Villand, P., Luthi E., Olsen, O.A., Preiss, J. (1993) "Insensitivity of barley endosperm ADP-glucose pyrophosphorylase to 3-phosphoglycerate and orthophosphate regulation" *Plant Physiol.* 101(1):179-86.

Lin, T., Caspar, T., Sommerville, C.R., Preiss, J. (1988) "A Starch Deficient Mutant of *Arabidopsis thaliana* with Low ADPglucose Pyrophosphorylase Activity Lacks One of the Two Subunits of the Enzyme" *Plant Physiol.* 88:1175-1181.

Maniatis, T., E.F. Fritsch, J. Sambrook (1982) "Nuclease *Bal*31" *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Morell, M., M. Bloon, V. Knowles, J. Preiss (1988) "Affinity Labeling of the Allosteric Activator Site(s) of Spinach Leaf ADP-glucose Pyrophosphorylase" *J. Bio. Chem.* 263:633.

Muller-Rober, B.T., J. Kossmann, L.C. Hannah, L. Willmitzer, U. Sounewald (1990) "One of Two Different ADP-glucose Pyrophosphorylase Genes From Potato Responds Strongly to Elevated Levels of Sucrose" *Mol. Gen. Genet.* 224:136-146.

Nakata, P.A., T.W. Greene, J.M. Anderson, B.J. Smith-White, T.W. Okita, J. Preiss (1991) "Comparison of the Primary Sequences of Two Potato Tuber ADP-glucose Pyrophosphorylase Subunits" *Plant Mol. Biol.* 17:1089-1093.

Okita, T.W., Nakata, P.A., Anderson, J.M., Sowokinos, J., Morell, J., Preiss, J. (1990) "The Subunit Structure of Potato Tuber ADPglucose Pyrophosphorylase" *Plant Physiol.* 93:785-90.

Okita, T.W., Greene, T.W., Laughlin, M.J., Salamone, P., Woodbury, R., Choi, S., Ito, H., Kavakli, H., Stephens, K. (1996) "Engineering Plant Starches by the Generation of Modified Plant Biosynthetic Enzymes," In *Engineering Crops for Industrial End Uses*, Shewry, P.R., Napier, J.A., and Davis, P., eds., Portland Press Ltd., London.

Olive, M.R., R.J. Ellis, W.W. Schuch (1989) "Isolation and Nucleotide Sequences of cDNA Clones Encoding ADP-glucose Pyrophosphorylase Polypeptides from Wheat Leaf and Endosperm" *Plant Physiol. Mol. Biol.* 12:525-538.

Ou-Lee, T. and Setter, T.L. (1985) "Effect of Increased Temperature in Apical Regions of Maize Ears on Starch-Synthesis Enzymes and Accumulation of Sugars and Starch" *Plant Physiol.* 79:852-855.

Preiss, J. (1984) "Bacterial Glycogen Synthesis and Its Regulation" *Ann. Rev. Microbiol.* 38:419-458.

Preiss, J. and Romeo, T. (1994) "Molecular Biology and Regulatory Aspects of Glycogen Biosynthesis in Bacteria" *Progress in Nuc. Acid Res. and Mol Biol.* 47:299-329.

Preiss, J. and Sivak, M. (1996) "Starch synthesis in sinks and sources," In *Photoassimilate distribution in plants and crops: source-sink relationships*. Zamski, E., ed., Marcil Dekker Inc. pp. 139-168.

Rijven, A.H.G.C. (1986) "Heat Inactivation of Starch Synthase in Wheat Endosperm Tissue" *Plant Physiol.* 81:448-453.

Shaw, J. R., Hannah, L. C. (1992) "Genomic Nucleotide Sequence of a Wild-Type Shrunken-2 Allele of *Zea mays*" *Plant Physiology* 98:1214-1216.

Singletary, G.W., Banisadr, R., Keeling, P.L. (1993) "Decreased starch synthesis in heat stressed maize kernels results from reduced ADPG-pyrophosphorylase and starch synthase activities" *Plant Physiol.* 102: 6 (suppl).

Singletary, G.W., Banisadra, R., Keeling, P.L. (1994) "Heat stress during grain filling in maize: effects of carbohydrate storage and metabolism" *Aust. J. Plant Physiol.* 21:829-841.

Smith-White, B. J. *et al.* (1992) "Comparison of Proteins of ADP-Glucose Pyrophosphorylase from Diverse Sources" *J. Mol. Evol.* 34:449-464.

Sowokinos, J.R., Preiss, J. (1982) "Pyrophosphorylases in *Solanum tuberosum*" *Plant Physiol.* 69:1459-1466.

Stark *et al.* (1992) "Regulation of the Amount of Starch in Plant Tissues by ADP Glucose Pyrophosphorylase" *Science* 258:287-292.

Thompson, L. M. (1986) "Climatic Change, Weather Variability, and Corn Production" *Agron. J.* 78:649-653.

Thompson, L. M. (1975) "Weather Variability, Climatic Change, and Grain Production" *Science* 188:535-541.

Tollenaar, M., Bruulsema, T. W. (1988) "Effects Of Temperature On Rate And Duration Of Kernel Dry Matter Accumulation Of Maize" *Can. J. Plant Sci.* 68:935-940.

Tsai, C.Y., Nelson, Jr., O.E. (1966) "Starch-Deficient Maize Mutant Lacking Adenosine Diphosphate Glucose Pyrophosphorylase Activity" *Science* 151:341-343.

Wilhelm, E. P., Mullen, R. E., Keeling, P.L., Singletary, G. W. (1999) "Heat stress during grain filling in maize: Effects on kernel growth and metabolism" *Crop Science* 39:1733-1741.

Xu, D., McElroy, D., Thornburg, R. W., Wu, R. *et al.* (1993) "Systemic induction of a potato pin2 promoter by wounding, methyl jasmonate, and abscisic acid in transgenic rice plants" *Plant Molecular Biology* 22:573-588.

Yang, T. T. *et al.* (1996) "Optimized Codon Usage and Chromophore Mutations Provide Enhanced Sensitivity with the Green Fluorescent Protein" *Nucleic Acid Research* 24(22):4592-4593.